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(54) **Steroid/protein conjugates**

(57) A method and composition for increasing ovulation in female cattle wherein the cattle are administered with the composition so as to produce a mean steroid binding antibody titre in the female cattle of from 1 in 100 to 1 in 5000 at the time of ovulation.

The composition comprises (i) a conjugate of a steroid androgen, such as androstenedione or testosterone, or of a steroid oestrogen, such as oestrone, and an immunogenic protein, such as human serum albumin, and (ii) acid anhydrides of the steroid derivative, the composition containing from 10 to 90% of the steroid moiety in the form of the anhydrides.

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SPECIFICATION
Control of ovulation

This invention relates to a process whereby the natural genetically determined ovulation rate of cattle may be manipulated and increased. In particular, it is concerned with immunization of cattle against androgenic and oestrogenic steroids in such a way that ovulation may be increased to a degree that significantly exceeds natural values. It is further concerned with the preparation and use of immunogenic compositions that can bring about the biological effects of the invention.

Because of their considerable economic importance, studies of the reproductive biology of domestic livestock have long been directed toward processes by which the individual fecundity of farm animals could be manipulated and in particular towards increasing the ovulation rate in females. The ovulations rate necessarily controls the maximum number of offspring that can be produced in a given pregnancy. Cattle have a very low natural ovulation rate, that is number of ovulations per animal occurring in the one oestrous cycle; for most breeds the ovulation rate lies between 1.00 and 1.01. Only in uncommon breeds are values such as 1.07 seen. Thus, cattle are very different in this aspect from many other domestic livestock species in which breeds often have ovulation rates of 2 or more. Clearly, for the natural fecundity of livestock to be increased, there must be at fertilization an increased ovulation rate.

It is already known in the prior art that, by artificially increasing the ovulation rate, in a domestic livestock species, by the administration of pregnant mare serum gonadotrophin (PMSG) for example, it is possible to increase the ovulation rate and ultimately the overall fecundity of the species. This process, however, suffers from the problem that the number of eggs shed cannot be controlled and the number of eggs shed by a cow treated with PMSG may vary from none up to 20.

In our copending Australian patent application No. PE 2274/80 it was also shown that by immunization of sheep and goats to produce antibodies against certain endogenous hormones, the ovulation rate of these animals could be increased. The hormones were steroids of the oestrogen and androgen classes. However, surprisingly, cattle have not responded to such immunizations with good antibody production nor with changes in ovulation rate when the same immunogens that promote ovulation rate increases in sheep and goats have been injected into the cattle.

The present invention consists in a method for increasing the ovulation rate in female cattle comprising administering to the cattle an immunogenic composition comprising (i) a conjugate of a steroid androgen derivative or a steroid oestrogen derivative with an immunogenic protein and (ii) acid anhydrides of the steroid derivatives, the composition containing from 10 to 90% of the steroid moiety in the form of the anhydrides, so as to produce a mean steroid binding antibody titre in the female cattle of from 1 to 100 to 1 in 5,000 at the time of ovulation.

In another aspect the present invention consists in a composition of matter efficacious in increasing the ovulation rate when administered to female cattle comprising (i) a conjugate of a steroid androgen derivative or of a steroid oestrogen derivative and an immunogenic protein and (ii) acid anhydrides of the steroid derivative, the composition containing from 10 to 90% of the steroid moiety in the form of the anhydrides.

As used in this specification, the following terms have meanings set out hereunder:

Steroid Androgen: Any steroid substance that stimulates the expression of secondary sex characteristics of the male. Androgenic activity can be assessed by measuring the regrowth of the involuted comb of a castrated cock following androgen administration or by measuring the growth response of the seminal vesicles in castrated male rates following androgen administration.

Steroid Oestrogen: Any steroid substance other than oestradiol-17β that stimulates the expression of secondary sex characteristics in the female. Oestrogenic activity can be assessed by measurement of uterine growth or cornification of the vaginal epithelium following oestrogen administration to spayed female rats or mice.

Immunizations against oestradiol-17β have been found to render female animals anoestrus due to the neutralization of its powerful hormonal effects and it is therefore not suitable for use in the present invention.

Antibody titre: Defined here as the dilution of the antiserum which binds 50% of the maximum amount of labelled steroid bound by the antiserum or by the standard reference quality control antiserum during incubation of about 50 picograms of steroid for about 18 hours at 4°C followed by the use of either dextran-coated charcoal or polyethyleneglycol to separate free from antibody-bound steroid.

The immunizations of the invention are carried out with substances and procedures which are individually known in the art but which in combination are novel. Thus, to render the androgens or oestrogens immunogenic, suitable acidic derivatives are synthesized and these are linked by chemical means, covalently, to an immunogenic protein such as human serum albumin, ovalbumin, gelatin or γ-globulins typically. To obtain effects on ovarian function and ovulation in cattle, it has been discovered, surprisingly, that the immunogens need to be prepared with particularly high levels of sterold incorporated into or associated with the carrier proteins. This is achieved by chemically reacting a large molar excess of steroid with the protein carrier. However, a high steroid incorporation alone is not a

sufficient property of the immunogen to provide the effects of the invention. Commonly, the excess steroid used to facilitate a high molar steroid incorporation is deliberately removed from the conjugate by dialysis against aqueous, or aqueous organic, solvents or by column chromatography procedures. We have found, surprisingly, that the immunogenic compositions that achieve the effects of the invention are obtained if such dialysis is not pursued to completion to remove all the dialysable substances. It is part of its novelty that isolation of the immunogenic composition is achieved simply by incomplete dialysis of the reaction mixture against water alone for a period of 8—24 hours, optimally, followed by lyophilization.

Chemical analysis of the immunogenic composition so formed reveals that it is comprised of covalent steroid-protein conjugate together with steroid acid anhydride loosely bound to protein and a small proportion of unreacted steroid derivative. Surprisingly, the presence of the steroid acid anhydrides particularly enhances the immunogenic response to the steroid protein complex (Table 1). We have found that to produce an enhanced response, the immunogen composition should contain 10—90% and preferably 50—80% of the steroid moiety in the form of the acid anhydride. The compositions of this invention possess immunogenicity that is substantially greater than those skilled in the art would expect from a knowledge of the covalently linked hapten content.

The chemical procedure used to link the androgen or oestrogen to immunogenic protein can be any of the procedures known in the art which will at the same time form the necessary armount of steroid acid anhydride. The immunogenic compositions so formed are administered to animals and the immune response to them potentiated with adjuvants such as DEAE-Dextran, the immunogen being in solution or suspension, or with the immunogen suspended rather than emulsified, in Freunds complete or incomplete adjuvant. Surprisingly, it has been found that emulsions of the immunogen in Freunds adjuvant are often poorly effective which may be because it is difficult to prepare an emulsion at the concentrations of immunogen required that is stable and remains stable for sufficiently long to produce the immunological responses after injection.

In preparation of the immunogenic compositions, substances already known in the art may be used. Thus, preferred androgen immunogens are prepared by the conjugation to protein of 4-androstene-3,17-dione derivatives functionalised with an acid group at positions 1, 7, 11 or 15 of the steroid ring, for example.

For immunization against testosterone, derivatives functionalized with an acid group at positions 3 or 17 may be used. The antibody titre range for androstenedione or testosterone that should be attained to achieve an increase in ovulation rate in cattle should preferably lie in the range 1:100 to 1:5000 as a mean for the herd. With antibody titres for these hormones which are significantly lower than this range, the incidence of multiple ovulations will tend to diminish and will approach that characteristic of unimmunized animals.

Suitable immunizations with the oestrogen, oestrone, can also promote an increase in the ovulation rate of cattle if titres lie in the range of 1 in 100 to 1 in 5,000 as a mean for the herd. The preferred steroid derivatives are those which, after conjugation with protein and administration to an animal, produce oestrone-specific antibody. Non-limiting examples of such oestrone derivatives already known in the art include oestrone-3-hemisuccinate, oestrone-3-carboxymethylether, oestrone-6-carboxymethyloxime and 15 carboxymethylestrone.

The invention places no limitation on the protein used to form the immunizing antigen of the invention but human serum albumin has been found effective.

The invention recognizes that the range of antibody titres may vary with the hormone used in the immunization and with the breed of cattle being immunized. The selection of the most preferred antibody titre within the above range is therefore a matter of experimentation in the particular circumstances of any application of this method.

The immunogenic composition is preferably administered by injections spaced apart by a period of from 1 to 5 weeks. In the case of an animal which has been previously immunised only a single injection may be required. It is considered desirable to allow one full oestrus cycle to pass between the immunization, or the second immunization if that occurs, and mating of the animal. The immunogenic material should therefore be such that the desired antibody titre is obtained upon ovulation in an oestrus cycle separated from the time of immunisation by at least the length of one complete oestrus cycle.

In the examples given the biological effects achieved are a consequence of the development of antisteroid antibodies because immunizations of control animals against the protein carrier alone had no significant effect on the ovulation rate. In applying these effects to the breeding of cattle it will be recognized by those skilled in the art that they may conveniently be combined with synchronization of the cycle, as with prostaglandins or progestagens, followed by artificial insemination, in which case there is no requirement for the cattle to show oestrus and recording of oestrus is not essential.

Further, they may conveniently be combined with both synchronization of oestrus and the use of luteinizing hormone releasing hormone, typically 66—90 hr after the last synchronization treatment, followed by artificial insemination 6—12 hr later again, in order to improve fertility through timing of ovulation as well as ensuring optimum ovulation rate.

The scope of this invention is not limited to processes employing active immunization protocols, for passive immunizations using antisteroid antibodies raised in donor animals may be used to achieve

the increases in ovulation rate encompossed by the invention.

All the prior art immunization technologies that may be used to achieve the critical antisteroid antibody titres and the biological response of the invention are embraced by its method and processes.

The following example is given to illustrate preferred methods within the broad scope of this invention.

EXAMPLE 1

(a) Preparation of steroid-protein immunogenic compositions

To an amount of 360 mg oestrone-3-carboxymethyl ether dissolved in 36 ml dioxane was added, dropwise and with stirring, a freshly prepared solution of 162 mg 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide hydrochloride (ECDI) dissolved in 10.8 ml distilled water. The reaction was allowed to proceed for 30 minutes at 25°C and then a solution of 360 mg human serum albumin (HSA) dissolved in 48 ml phosphate buffer pH 7.80, 0.05M was added dropwise with stirring. After 18 hr a further solution of 180 mg oestrone-3-carboxymethylether in 12 ml dioxane was mixed with 54 mg ECDI in 3.6 ml water, allowed to stand 30 minutes at 25° and added to the main reaction mixture. The reaction was allowed to continue for $4\frac{1}{2}$ hr and then a further 108 mg ECDI was added in solid form directly and with stirring to the mixture.

After a further 3 hr at 25°C the reaction mixture was transferred to dialysis tubing and dialysed against distilled water. The water was changed after about 1, $1\frac{1}{2}$, 2 and 12 hr. The product (oestrone immunogenic composition) retained in the dialysis sack was then lyophilized and weighed. Yield 400 mg. The steroid content of the steroid-protein immunogen was calculated by incorporating a trace amount of ^{3}H-labelled oestrone-3-carboxymethylether in the reaction. By liquid scintillation counting of weighed amounts of the steroid derivative and the immunogen it was found that 120 moles steroid equivalent per mole protein were present in the immunogenic composition; 35 moles steroid/mole protein were covalently linked, the remainder being steroid acid anhydride loosely bound to protein.

In a similar preparation 7α-carboxyethylthio-4-androstene-3,17-dione was linked to human serum albumin and yielded 380 mg of product (androstenedione immunogen composition having 90 moles steroid equivalent per mole protein present of which 32 moles were covalently linked and the remainder being steroid anhydride loosely bound to the protein.

In a similar preparation testosterone-3-carboxymethyloxime was linked to human serum albumin and yielded 385 mg of product (testosterone immunogenic composition) having 102 moles steroid equivalent per mole of protein present, of which 33 moles were covalently linked, the remainder being steroid acid anhydride loosely bound to the protein.

(b) Immunization of cattle to test steroid immunogenic composition

100—250 mg of oestrone, androstenedione or testosterone immunogenic compositions were pasted in a 1 ml 0.9% sterile saline and made into a total volume of 15 ml 0.9% sterile saline. Then 15 ml DEAE-dextran solution was added. The DEAE dextran solution was prepared by dissolving 15 g in 100 ml water and adjusting the pH to 7.5—7.7 using saturated tri-(hydroxy-methyl)-methylamine buffer (500 g/litre water). The final volume of the DEAE dextran solution was then adjusted to 150 ml.

Alternatively, 100—250 mg of steroid immunogenic compositions were pasted in 1 ml Freunds adjuvant and made into a suspension in a total volume of 30 ml in Freunds adjuvant.

Cattle (of Hereford and Hereford-Friesian breeds) were injected each with 3 ml of the vaccine, containing 10 or 25 mg immunogen, each animal being given injections at about 10 sites subcutaneously over the neck region. An intramuscular injection of 1.5 ml pertussis vaccine was also administered. The injection treatment was repeated 4 weeks later.

Blood samples were taken by tail vein puncture one week after the second treatment. Blood was collected into heparinized tubes, stored on ice and centrifuged at 4°C. The plasma so obtained was stored at −10°C until analysis of steroid antibody titre. The steroid-antibody titres obtained from the immunogenic compositions in (a) are shown in Table 1, together with the low values obtained with conventionally prepared steroid-protein conjugates.

EXAMPLE 2

Immunization of cattle against steroids and effect of ovarian size and activity

Using immunogenic compositions and methods as described in Example 1 cattle were treated and examined. Ovarian changes were assessed by palpation per rectum in the period 8—17 weeks after commencing treatment and the number and size of follicles or corpora lutea on left and right ovaries were noted. Occurrence of oestrus in the cattle was determined using K-Mar heat mount detectors; cattle generally continued to show oestrus following treatment.

The result of immunization are given in Table 2 showing marked increase in ovarian activity, follicle number and numbers of corpora lutea with immunization against oestrone and androstenedione compared to control animals.

TABLE 1
Enhanced immunogenicity of Composition Containing Steroid Acid Anhydrides in Addition to Steroid Protein Conjugates

Steroid Derivative used in making Immunogen Composition	Total Steroid Moiety Present	Covalently Linked Hapten Content	Number of Animals Treated	Mean Steroid Antibody Titre Produced* for Group
	Moles/Mole Protein			
Testosterone-3-carboxymethyl oxime	33≠	33	5	Below 1:50
	102+	33	8	1:865
Androstenedione-7α-carboxyethyl thioether	33≠	33	6	Below 1:50
	90+	32	6	1:300
Oestrone-3-carboxymethylether	120+	35	6	1:6420

* Following 2 treatments — primary and secondary
+ These preparations contain a high proportion of steroid acid anhydrides
≠ Preparations of steroid-protein conjugate conventionally dialysed to completely remove excess steroid acid anhydrides.

TABLE 2
Ovarian Changes in Cattle 11 Weeks after Start of Treatment

Steroid Derivative used in making Immunogenic Composition	Number of Animals	Animals with enlarged ovaries	Animals with multiple corpora lutea or several large follicles on the ovaries
Oestrone-3-carboxymethylether	9	5	5
Androstenedione-7α-Carboxyethylthioether	6	4	2
Controls (Untreated)	10	1	0

EXAMPLE 3

Using immunogenic compositions and methods as described in Example 1 to maintain titres but including repeated treatments a further series of tests with immunizations against testosterone, androstenedione or oestrone was carried out. Palpation per rectum indicated ovarian responses and these were then confirmed and detailed by endoscopic examination of the cattle (Table 3) in which the ovaries were visualized and size, number of follicles and corpora lutea were recorded.

TABLE 3
Endoscopic Examination of Cattle Following Immunization Against Steroids and Showing Resultant Increases in Ovulation Rate

Immunization Against Steroid	Number of Animals in Groups	Mean Steroid Antibody Titre	Number of Animals having 2 or 3 Ovulations
Testosterone	7	270	3
Androstenedione	6	300	2
Oestrone	8	1500	1
Total of Immunized Animals	21	—	6 (29%)
Control (reference)	2	0	0

Only a small control group was run in this series as it is well established that the multiple ovulation rate in untreated cattle of the Hereford or Hereford-Friesian breeds is very low and about 1% i.e. the untreated cattle have an ovulation rate of 1.01 as compared with the ovulation rate of the total of immunized animals in this example of 1.29.

CLAIMS

1. A method for increasing the ovulation rate in female cattle comprising administering to the cattle an immunogenic composition comprising (i) a conjugate of a steroid androgen derivative or a steroid oestrogen derivative with an immunogenic protein and (ii) acid anhydrides of the steroid derivatives, the composition containing from 10 to 90% of the steroid moiety in the form of the anhydrides, so as to produce a means steroid binding antibody titre in the female cattle of from 1 in 100 to 1 in 5,000 at the time of ovulation.

2. A method as claimed in Claim 1 in which the immunogenic composition contains from 50 to 80% of the steroid moiety in the form of the anhydride.

3. A method as claimed in Claim 1 or Claim 2 in which the steroidal androgen is selected from the group comprising 4-androstene-3,17-dione and testosterone.

4. A method as claimed in Claim 1 or Claim 2 in which the steroidal oestrogen is oestrone.

5. A method as claimed in any one of Claims 1 to 4 in which the immunogenic protein is selected from the group comprising human serum, albumin, ovalbumin, gelatin and γ-globulin.

6. A method as claimed in any one of Claims 1 to 5 in which the conjugate contains 25 to 35 moles of steroid androgen or oestrogen derivative per mole of immunogenic protein.

7. A method as claimed in any one of Claims 1 to 6 in which the immunogenic composition is administered together with an Immunoadjuvant.

8. A method as claimed in Claim 7 in which the immunoadjuvant is selected from the group comprising diethylaminoethyldextran, Freunds complete adjuvant and Freunds incomplete adjuvant.

9. A method as claimed in Claim 3 in which the steroid androgen derivative is selected from the group comprising 4-androstene-3,17-dione-7α-carboxyethylthioether and testosterone-3-carboxymethyloxime.

10. A method as claimed in Claim 4 in which the steroid oestrogen derivative is oestrone-3-carboxymethylether.

11. A method as claimed in any one of Claims 1 to 10 in which oestrus in the cattle is synchronized with prostaglandins or progestagens and in which the cattle are artificially inseminated from 72 to 96 hours after the administration of such prostaglandins of progestagens.

12. A method as claimed in any one of Claims 1 to 10 in which oestrus in the cattle is synchronised with prostaglandins or progestagens; and in which luteinizing hormone releasing hormone is administered to the cattle from 66 to 90 hours thereafter and the cattle are artificially inseminated from 6 to 12 hours after the administration of the luteinizing hormone releasing hormone.

13. A method for increasing the ovulation rate in female cattle comprising passively administering to the cattle antibody raised in donor animals by a method as claimed in any of Claims 1 to 12 so as to provide a mean steroid binding antibody titre in the female cattle to form from 1 in 100 to 1 in 5,000 at the time of ovulation.

14. A composition of matter efficacious in increasing the ovulation rate when administered to female cattle comprising (i) a conjugate of a steroid androgen derivative or of a steroid oestrogen derivative and an immunogenic protein and (ii) acid anhydrides of the steroid derivative, the composition containing from 10 to 90% of the steroid moiety in the form of the anhydrides.

15. A composition as claimed in Claim 14 in which from 50 to 80% of the steroid moiety is in the form of the anhydride.

16. A composition as claimed in Claim 14 or Claim 15 in which the steroidal androgen is selected from the group comprising 4-androstene-3,17-dione and testosterone.

17. A composition as claimed in Claim 14 or Claim 15 in which the steroidal oestrogen is oestrone.

18. A composition as claimed in any one of Claims 14 to 17 in which the immunogenic protein is selected from the group comprising human serum, albumin, ovalbumin, gelatin and γ-globulin.

19. A composition as claimed in any one of Claims 14 to 18 in which the conjugate contains 25 to 35 moles of steroid androgen or oestrogen derivative per mole of immunogenic protein.

20. A composition as claimed in any one of Claims 14 to 19 in which the composition also contains an immunoadjuvant.

21. A composition as claimed in Claim 20 in which the immunoadjuvant is selected from the group comprising diethylaminoethyldextran, Freunds complete adjuvant and Freunds incomplete adjuvant.

22. A composition as claimed in Claim 16 in which the steroid androgen derivative is selected from the group comprising 4-androstene-3,17-dione-7α-carboxyethylthioether and testosterone-3-carboxymethyloxime.

23. A composition as claimed in Claim 17 in which the steroid oestrogen derivative is oestrone-3-carboxymethylether.

24. A method for increasing the ovulation rate in female cattle substantially as hereinbefore described with reference to Examples 1 to 3.

25. A composition of matter efficacious in increasing the ovulation rate when administered to female cattle substantially as hereinbefore described with reference to Examples 1 to 3.

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